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ROLES OF THE MAMMALIAN CYTOSOLIC CYSTEINE DESULFURASE, ISCS, AND SCAFFOLD PROTEIN, ISCU IN IRON-SULFUR CLUSTER ASSEMBLY

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Running title: Cysteine desulfurase activity of mammalian cytosolic ISCS

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Iron-sulfur clusters are prosthetic groups composed of sulfur and iron that are found in respiratory chain complexes and numerous enzymes. Iron-sulfur clusters are synthesized in a multi-step process that utilizes cysteine desulfurases, scaffold proteins, chaperones, and iron donors. Assembly of iron-sulfur clusters occurs in the mitochondrial matrix of mammalian cells, but cytosolic isoforms of three major mammalian iron-sulfur cluster assembly components have been found, raising the possibility that *de novo* iron-sulfur cluster biogenesis also occurs in cytosol. The human cysteine desulfurase, ISCS, has two isoforms, one of which targets to the mitochondria, whereas the other less abundant form is cytosolic and nuclear. The open-reading frame of cytosolic mammalian ISCS begins at the second AUG of the transcript and lacks mitochondrial targeting information. Yeast complementation experiments have suggested that the human cytosolic ISCS isoform (c-ISCS) cannot be functional. To evaluate function of c-ISCS, we overexpressed the human cytosolic ISCS in yeast *Pichia pastoris*, and showed that the cytosolic form of ISCS is an active cysteine desulfurase that covalently binds ^{35}S acquired from desulfuration of radiolabeled cysteine. Human cytosolic ISCS dimerized as efficiently as bacterial ISCS and formed a complex *in vitro* with over-expressed cytosolic human ISCU. When incubated with iron regulatory protein 1, cysteine and iron, the cytosolic forms of ISCS and ISCU facilitated efficient formation of a [4Fe-4S] cluster on IRP1. Thus, the cytosolic form of ISCS is a functional cysteine desulfurase that can collaborate with cytosolic ISCU to promote *de novo* iron-sulfur cluster formation.

Iron-sulfur clusters are prosthetic groups required for function of numerous enzymes and respiratory chain complexes. Enzymes involved in iron-sulfur cluster biogenesis were first identified in bacteria, and homologues have since been found in plants, animals, and fungi (reviewed in (1)). In the human genome, homologues of the cysteine desulfurase (2), the scaffold proteins ISCU (3) and NFU (4), an Hsc20 chaperone (5) and other iron-sulfur cluster assembly enzymes are present. Interestingly, isoforms of ISCS, ISCU and NFU are found in both mitochondria and cytosol, and ISCS and ISCU form a distinct complex in mammalian cytosol (3), strongly suggesting that there is compartmentalization of Fe-S cluster biogenesis in mammalian cells. In contrast, mitochondria are believed to be the sole site of iron-sulfur cluster assembly in yeast (6). In *Saccharomyces cerevisiae*, a single cysteine desulfurase with high homology to ISCS is encoded by Nfs1p. Nfs1p was initially detected only in mitochondria (7), but there is also a cytosolic/nuclear form of yeast NFS1 (8), which is required for cell viability and for post-transcriptional modification of tRNAs in the nucleus and mitochondria (9)(10).

A single ISCS homologue identified in the human genome generates two distinct isoforms through alternative utilization of in-frame AUGs (2). The major ISCS isoform (m-ISCS) generated by initiation at the first AUG of the ISCS transcript contains a mitochondrial targeting sequence at the N-terminus that undergoes cleavage to yield a mature mitochondrial protein of 47 kDa in size. A less abundant isoform generated by initiation of translation at the second in-frame AUG lacks the first 60 residues of the mitochondrial precursor form, and this 44kDa cytosolic ISCS (c-ISC-S) resides in both the cytosol and nucleus (2). A highly conserved tyrosine residue (Y60 of human m-ISCS, which is equivalent to Y101 of *S.cerevisiae* Nfs1) is absent in c-ISCS. Recently, it was reported that the removal of 101 amino acids from the N-terminus of yeast Nfs1 to approximate the N-terminal sequence of human cytosolic ISCS inactivated cysteine desulfurase activity in a yeast growth restoration assay, whereas removal of the only first 95 amino acids yielded a functional enzyme (see sequence alignments in Fig 1). These results led to the proposal that Y60 is essential for dimerization and enzymatic function of ISCS, and were further interpreted as proof that human cytosolic ISCS (c-ISCS) could not be a functional enzyme (10).

To directly assess whether the cytosolic form of ISCS can function, we over-expressed human c-ISCS in *Pichia pastoris* and assessed its cysteine desulfurase activity. We demonstrate that c-ISCS is a fully functional cysteine desulfurase that dimerizes as efficiently as the IscS of *Azotobacter vinelandii*, binds to human cytosolic ISCU (c-ISCU) in a complex, and facilitates assembly of an iron-sulfur cluster on iron regulatory protein 1 (IRP1). We found that the cytosolic form of ISCU was equally as efficient as the mature mitochondrial form of ISCU in facilitating reconstitution of the iron-sulfur cluster of IRP1. Thus, human c-ISCS and c-ISCU are fully functional, and their presence in cytosol is physiologically relevant.

Experimental Procedures

Constructs – ISCS and ISCU were overexpressed in *Pichia* under the control of the strong alcohol oxidase promoter *(AOX1)*. For over-expression of c-ISCS in *Pichia*, PCR products were generated with primers P1 5′-GAGGATCCA**CCATGG**ATGTGCAAGCTA-3′ and P2 5′-CGG**GAATTC**TAGTGTTGGGTCCACTTG-3′ and the cDNA sequence of human ISCS derived from EST clones. The *Bam*HI-*Eco*RI restriction enzyme-generated fragment of the *isc*S PCR product was cloned into the expression vector pPIC3.5K (Invitrogen, Carlsbad, CA) to generate pPIC-cIscS for overexpression in *Pichia* SMD1168 (Invitrogen). The construct pPIC-cIscS was also transformed into DS115(CA13-02) (11) to generate DS115(c-ISCS+IRP1) for co-over-expression of c-ISCS and IRP1.

For over-expression of c-ISCU in *Pichia*, PCR products were generated with primers P3 5′-GGGGATCCATGGTTCTCATTGAC-3′ and P4 5′-GCGCCTCGAGAGGGAGGGCTCATT-3′ and an EST clone EST73531 that contains the coding sequence of cytosolic ISCU (3). The *Bam*HI-*Xho*I fragment of *isc*U PCR product was cloned into pET24a⊕ (Strategene, La Jolla, CA) to generate pET-cIscU for overexpression in *E. coli* BL21(DE3), or into pCA10.3 (11) to generate pCA-cIscU for overexpression in *Pichia* SMD1168 (Invitrogen). For overexpression of m-ISCU in BL21(DE3), primers P5 5′-GGGGCATATGGCATATCACAAGAAGGTTGTT-3′ and P6 5′-GGGCTCGAGTTTCTTCTCTGCCTCTCCTTT-3′ were used to amplify mature form of m-*isc*U by PCR. *Nde*I-*Xho*I PCR product was cloned into pET24a⊕ to get pET-mIscU. Over-expressed c-ISCU and m-ISCU in *E. coli* were C-terminal His-tagged and under the control of the *lac* transcriptional elements. To over-express the mature form of m-ISCU we added a start code ATG, followed by GCA, which encodes the amino acid ‘A’, followed by the sequence

TAT, which encodes the 35th amino acid 'Y', the first amino acid distal to the predicted cleavage site of the mitochondrial target sequence in the N-terminus. The remainder of the sequence was as written for Hs m-ISCU in the alignment of Fig. 1B. Nucleotide sequences for all constructs were confirmed by DNA sequencing.

Overexpression and purification of human cytosolic and mitochondrial ISCU, cytosolic ISCS and IRP1 - For overexpression of c-ISCU and m-ISCU, *E. coli* strain BL21(DE3) cells harboring pET-cIscU or pET-mIscU was cultured in 2-liter flasks containing 700 ml of Terrific medium (Research Products International corp., Mt. prospect, IL) supplemented with kanamycin (50 mg/l) in a shaker at 37 °C/250 rpm until the optical density at 600nm reached 0.8. Expression of c-*isc*U or m-*isc*U was induced by the addition of isopropyl thio-β-d-galactoside (IPTG) to a final concentration of 0.5 mM. After induction, bacteria were cultured for 3 h and harvested by centrifugation, and the cell pellets were frozen at -70 °C until used. The frozen cells were thawed in a lysis buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, and 10 mM imidazole) and subsequently sonicated and centrifuged. The soluble c-ISCU or m-ISCU protein was purified by affinity chromatography using Ni-nitrilotriacetic acid gel recommended by manufacturer (Qiagen, Valencia, CA). Notably, c-ISCS could not be over-expressed and purified from bacteria because it was sequestered in inclusion bodies.

Details about the overexpression of IRP1 in *P. pastoris* under the control of the strong alcohol oxidase promoter *(AOX1)*, as well as protein purification, were described previously (11). In the last step of the purification, an affinity column of IRE (iron regulatory element)-binding was used to isolate the apo-IRP1. Human cytosolic ISCS and ISCU were overexpressed in *P. pastoris* following procedures similar to those used for IRP1 overexpression. For purification of c-ISCS, the harvested and lysed cells were centrifuged at 31000*g* for 15 min. Solid $(NH_4)_2SO_4$ was added to the supernatant to 15% saturation. Precipitated protein was removed by centrifugation and resuspended in 16 ml of 0.05 M Tris-HCl, pH 8, 50 mM $(NH_4)_2SO_4$, loaded onto a column of DEAE Sephadex (5 ml), and equilibrated with Buffer A (50 mM Tris, pH8.0). The flowthrough was loaded onto a Heparin sulfate column and protein was eluted from the column at 1 ml/min with a linear gradient of buffer A and buffer B (50 mM Tris pH8.0, 0.5 M NaCl), and subsequently concentrated with an Amicon concentrator (Millipore, Billerica, Massachusetts). The purity of the recombinant proteins was checked by SDS/PAGE after each step of the purification. The protein concentration was determined with Bradford assay.

Demonstration of in vitro complex formation between c-ISCU and c-ISCS – Pichia cells expressing c-ISCS or c-ISCU were lysed in 2 ml of lysis buffer (25 mM TrisHCl, pH8.0, 100 mM NaCl, 2.5 μg/ml aprotinin, 5 μg/ml leupeptin, 0.4 mM phenylmethylsulfonyl fluoride, and 5 mM benzamidine). After cells were broken with the one-shot cell disrupter (Cell Disruption System, St-Laurent, Quebec, Canada), the lysate was centrifuged at 27000*g* for 30 min. The supernatants containing overexpressed c-ISCS or c-ISCU were either mixed together or separately incubated on ice for 3 hours. One ml of mixed or non-mixed lysate was loaded on a HiPrep^{TM} 16/60 sephacryl^{TM} S-300 FPLC column (Amersham-Pharmacia Biotech Inc. Piscataway, NJ) and equilibrated with elution buffer (25 mM TrisHCl, pH8.0, 100 mM NaCl). The column was eluted with 1.5 bed volumes of elution buffer. Successive 1 ml fractions were collected and applied to 12% SDS-PAGE gel for analysis of c-ISCU and c-ISCS distribution by Western blot. Since we could not observe the monomer or dimmer size of c-ISCS from the yeast lysate containing overexpressed c-ISCS by the size exclusion column (SEC), we used the purified c-ISCS to run SEC. The condition is the same as described above for the lysate. For HMW gel filtration calibration, the standard proteins (Pharmacia,

Piscataway New Jersey) were used, which include thymoglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), albumin (67 kDa), chymotrypsinogen (25 kDa), and cytochrome C (12.3 kDa).

Enzymatic assays – ISCS desulfurase activity was determined using a sulfide detection assay described previously (12). The amount of c-ISCS in lysate was determined by comparison to a concentration curve of purified protein, background activity from yeast into which an empty plasmid was transformed was subtracted, and the specific activity was calculated in nmol[S^{-2}]/mg/min. Alternatively, ISCS desulfurase activity was demonstrated using a ^{35}S transfer assay. Sulfur transfer reactions were carried out at 23 °C for 30 s in an assay containing 1.6 μM ISCS, 50 mM Tris, pH 7.5, 40 mM KCl, 10 mM $MgCl_2$ in a final volume of 30 μl. Reactions were initiated by the addition of ^{35}S-labeled L-cysteine (0.19 Ci/mmol, Amersham Pharmacia Biotech) to a final concentration of 15 μM and were terminated by centrifugation at 2000 × *g* through a size exclusion column (Microspin G-50 column; Amersham Pharmacia Biotech Inc.). The spin column eluant was mixed with sample loading buffer to give final concentrations of 1% glycerol, 5 mM Tris, pH 8.0, and 0.001% bromophenol blue. Samples were immediately analyzed by electrophoresis in BupH™ Tris-HEPES-SDS gel (PIERCE, Rockford, IL), but in the absence of added reducing agent and no heating. ^{35}S was visualized by a PhosphoImager (Pharmacia, Typhoon 9200)

Fe-S cluster assembly was monitored by assaying aconitase activity using an in-gel assay or two different spectrophotometric assays. The in-gel aconitase assay was performed as described previously (13). Overall aconitase activity of cell lysate was assayed by a coupled assay in which MTT reduction was monitored by the increase of absorbance at 570 nm at 22°C in an assay containing 2 mM *cis*-aconitic acid, 1 mM $NADP^+$, 25 mM $MgCI_2$, 100 mM potassium phosphate, pH 6.5, 1-2 units of isocitrate dehydrogenase (IDH), 1.2 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 0.3 mM phenazine methosulfate (PMS), and extract in a total volume of 1.0 ml. After Fe-S cluster reconstitution with purified c-ISCS and c-ISCU and transfer of Fe-S cluster to apo-IRP1, aconitase activity was followed by monitoring $NADP^+$ reduction in an assay containing cis-aconitate (2 mM), and 2 units of IDH, 0.2 mM $NADP^+$, and 0.6 mM $MnCl_2$ in a volume of 400 μl. The kinetics of NADPH production was measured at 340 nm assuming an extinct co-efficient of 6200 $M^{-1}cm^{-1}$. Aconitase activity was calculated in μmol [NADPH]/min. Based on this aconitase activity, converted IRP1 (nmol) was calculated by referring to the specific activity of beef liver IRP1 (3.4 U/nmol[4Fe-4S]) when the substrate cis-aconitate was used (14). To calculate the percentage of apo-IRP1 that converts to holoprotein, stoichiometry of one [4Fe-4S] per monomer was assumed and the amount of initial apo-IRP1 was set to 100%.

Reconstitution of Fe-S clusters in IRP1 - Each 2-ml Fe-S cluster assembly mixture included a 25 mM Tris-HCl, 40 mm KCl buffer (pH 7.4) that contained 50 µM c-ISCU, 5 µM c-ISCS, 2 mM L-cysteine, 0.2 mM ferric ammonium citrate, 5 mM DTT, 10 µM pyridoxal phosphate (PLP). For control experiments c-ISCU and/or c-ISCS were omitted from the assembly mixture. All assembly reactions were incubated in an anaerobic chamber for 3 h and then applied to a prepacked Mono Q™ 5/50 GL (Amersham Biosciences) previously equilibrated with 25 mM Tris-HCl (pH8.0) buffer. After loading, the sample was washed with 10 ml of the same buffer, which removes free L-cysteine. Proteins were then eluted sequentially using 200 mM followed by 400 mM NaCl in 25 mM Tris-HCl (pH8.0) buffer. Free Fe, S^{2-}, and polymeric iron sulfides remained bound to the column. The eluted fractions were then incubated for 30 min with 10 µl of 10 µM purified apo-IRP1. Aconitase activity was determined using either the in-gel assay as described in (13) or a spectrophotometric assay to evaluate conversion of apo-IRP1 to the [4Fe-4S]-IRP1 form.

RESULTS

To characterize the activity of cytosolic isoforms of human c-ISCS and c-ISCU (previously hIscU1 in ref (3), c-ISCS and c-ISCU were over-expressed in *Pichia pastoris* under the control of the AOX1 promoter for high-level, methanol-inducible expression. The cytosolic isoforms of human ISCS and ISCU differ from the mitochondrial isoforms at the N-terminus, as shown in figure 1, but mitochondrial and cytosolic isoforms of ISCS and ISCU are identical throughout the rest of the protein. Western blot analysis showed over-expression of human c-ISCU, c-ISCS and/or IRP1 proteins in *Pichia* after induction with methanol (Fig. 2A). Purified c-ISCS was obtained by ammonium sulfate precipitation, followed by DEAE column purification and elution from a heparin sulfate column (Fig. 2*B*).

Previously, protein complexes containing homologues of ISCS and ISCU have been observed in bacteria, yeast and mammalian cells (3) (15) (16) (17) (18), suggesting that specific physical interactions between these proteins may be crucial for *in vivo* Fe-S cluster biogenesis. To assess whether c-ISCS and c-ISCU can form a complex *in vitro*, we examined the elution profiles of c-ISCS and c-ISCU on a FPLC size exclusion column. Western blot analysis showed that c-ISCS or c-ISCU eluted in markedly different fractions due to their different molecular weights. However, when lysates containing c-ISCS and c-ISCU were mixed prior to fractionation, both proteins were detected in fractions 31-39 (Fig. 3), indicating that a protein complex of c-ISCS and c-ISCU formed *in vitro*. In contrast, purified c-ISCS migrated in a position consistent with the size of a monomer (43 kDa) in fraction 75, that also may form dimers (86 kDa) in fractions 67- 71, but does not comigrate with c-ISCS in yeast lysate, implying that c-ISCS forms a large protein complex with *Pichia* proteins. Our results suggest that c-ISCS and c-ISCU are capable of forming a protein complex in vitro.

To evaluate cysteine desulfurase activity of c-ISCS, enzymatic activity was measured using an assay based on ability of the enzyme to generate hydrogen sulfide (12). The sulfide was detected after converting it to methylene blue in an acidified solution of N, N-dimethylenediamine and $FeCl_3$. The cysteine desulfurase activity markedly increased in lysates of yeast that over-expressed c-ISCS (Fig. 4*A*), compared with lysates from strains transformed with empty plasmid or IRP1. Purified protein and activity analysis of four separate experiments indicated a specific activity of 6.4 ± 0.4 nmol/mg/min. In comparison, analysis of purified *A. vinelandii* NifS (a generous gift of Dennis Dean) yielded a specific activity of 75 nmol/mg/min. The previously published specific activity for *S. cerevisiae* is 12 nmol/mg/min. Thus, the specific activity of c-ISCS is comparable to *S. cerevisiae* Nfs1 (10), and both are lower than the reported specific activity of NifS (19).

Dimerization of ISCS is thought to be important in its enzymatic function (10) (20) and we therefore separated purified ISCS on a non-reducing gel with or without addition of GSH to evaluate whether c-ISCS can dimerize. In the absence of reductant, both monomeric and dimeric forms of c-ISCS were detected by Coomassie staining (Fig 4*B*). However, addition of GSH reduced but did not eliminate the upper band between the 64 and 98 kD markers, consistent with the possibility that the upper band represents a dimer that is further stabilized by disulfide bond formation between monomers.

In the first step of Fe-S cluster assembly, ISCS acquires sulfur by converting cysteine to alanine and binding sulfur in a persulfide. To evaluate the ability of ISCS to form the persulfide using cysteine as a substrate, a ^{35}S transfer assay was performed. Both the monomeric and dimeric forms acquired ^{35}S labeling from ^{35}S labeled cysteine (Fig. 4*B*), indicating that c-ISCS can function as a cysteine desulfurase. To further evaluate the efficiency of dimerization and cysteine persulfide formation, c-ISCS was compared to the IscS from *A. vinelandii*. Dimerization and radioactive cysteine persulfide formation of c-ISCS were

observed to be at least as efficient as the *A. vinelandii* IscS (Fig. 4*C*).

Three different types of Fe-S cluster biosynthetic systems have been discovered thus far, and all of them are mechanistically unified by the requirement for a cysteine desulfurase, such as IscS, and the need for a Fe-S cluster scaffold protein, such as IscU, in *de novo* iron-sulfur cluster assembly. To evaluate the potential of c-ISCS and c-ISCU to facilitate formation of iron-sulfur clusters, the ability of these enzymes to provide iron-sulfur clusters to aconitase was evaluated by an in-gel aconitase assay (13). Control yeast cells transformed with empty plasmid exhibited endogenous m-aconitase activity, which was not significantly enhanced by over-expression of human cytosolic ISCS or ISCU (Fig. 5*A*). Interestingly, IRP1 showed some aconitase activity when it was over-expressed in the cytosol of *P. pastoris* (Fig. 5*A*), but although we estimate that over-expressed IRP1 was as least 100-fold more abundant than mitochondrial aconitase, (11) and data not shown), the aconitase activities of endogenous mitochondrial aconitase and over-expressed IRP1 were approximately equal, indicating very inefficient conversion of over-expressed apo-IRP1 to its aconitase form in *P. pastoris* cytosol. We then asked if c-ISCS or c-ISCU could activate IRP1 aconitase activity, by adding lysates containing either c-ISCS or c-ISCU to lysates containing IRP1. Aconitase gel assays showed that addition of lysates containing c-ISCS or c-ISCU had only a minor effect on the aconitase activities of IRP1 or yeast m-aconitase, but simultaneous addition of c-ISCS and c-ISCU over-expression lysates led to a marked increase in IRP1 aconitase activity (Fig. 5*B*), suggesting that c-ISCS and c-ISCU cooperated to facilitate Fe-S cluster assembly in IRP1. Similarly, mitochondrial aconitase released from the mitochondria during lysate preparation also acquired Fe-S cluster in the presence of c-ISCS and c-ISCU and exhibited increased enzymatic activity (Fig. 5*B*).

Since acquisition of the iron-sulfur clusters of both apo-IRP1 and mitochondrial aconitase was facilitated *in vitro* by the scaffold protein c-ISCU (Fig. 5*B*), we compared the efficiency of c-ISCU in iron-sulfur cluster reconstitution to the efficiency of over-expressed m-ISCU, which was designed to closely match the mature mitochondrial form of ISCU predicted to function after mitochondrial import, processing and cleavage (described in materials and methods). The relative reconstitution efficiencies of c-ISCU and m-ISCU were evaluated by an in-gel assay at three different time points performed in duplicate (Fig. *5C*). As indicated in figure 5C, cytosolic ISCU and mature mitochondrial ISCU were almost equally efficacious as scaffold proteins in Fe-S cluster assembly.

To test whether c-ISCS and c-ISCU can assemble and transfer a Fe-S cluster to IRP1 in the absence of other factors present in yeast lysate, c-ISCS and c-ISCU were purified as described in experimental procedures and incubated with iron, DTT and cysteine in an anaerobic chamber for 3 hrs to reconstitute the Fe-S cluster in c-ISCU. Control experiments were performed in the absence of one or both proteins. Each reaction mixture was then applied to a Mono^{TM}-Q column and proteins were eluted with increasing concentrations of NaCl. Apo-IRP1 was then added to each eluate fraction and the fractions were incubated for an additional two hours in the anaerobic chamber. Aconitase activity of each eluate fraction was evaluated by the in-gel aconitase assay. The experimental scheme is outlined in Fig. 6*A*. No significant activation of IRP1 aconitase activity was observed when c-ISCS and c-ISCU were absent or only c-ISCU was absent while a very small activation was observed when c-ISCU was present (Fig. 6B, top three panels). IRP1 aconitase activity was significantly reconstituted when both c-ISCS and c-ISCU were present in the initial reaction (Fig. 6B, 4th horizontal panel). The minimal activation of IRP1 aconitase in the c-ISCU- only panel indicates that c-ISCU might be a functional scaffold protein since the Fe-S cluster can chemically assemble *in vitro*. The significant activation of IRP1 that occurs when both c-ISCS and c-ISCU

are present strongly suggests that the Fe-S cluster assembly on c-ISCU is mediated by c-ISCS. To determine whether the Fe-S cluster of activated IRP1 aconitase was transferred from the scaffold protein c-ISCU, we identified which Mono-Q eluate fractions contained c-ISCU by immunoblotting. IRP1 aconitase activity was acquired only from fractions that contained c-ISCU (Fig. 6*C, top two panels*), even though equal amounts of apo-IRP1 protein were added to each fraction (Fig. 6*C, bottom panel*), indicating that IRP1 acquired an Fe-S cluster only when reconstituted c-ISCU was present. These results are consistent with c-ISCS-mediated assembly of a Fe-S cluster in c-ISCU that can be transferred from the reconstituted c-ISCU to IRP1.

In addition to using the in-gel activity assay to evaluate ISCS/ISCU-mediated activation of IRP1, aconitase activity was also assessed with the $NADP^+$ reduction spectrophotometric assay. In this coupled assay, active IRP1 aconitase converts cis-aconitate to the isocitrate dehydrogenase substrate, isocitrate, which then undergoes conversion to alpha-ketoglutarate with concomitant reduction of $NADP^+$ to NADPH, which is measured by absorbance at 340 nm. The coupled aconitase assay indicated that aconitase activity was much greater (>9 fold) in IRP1 incubated with reconstituted c-ISCU than in the control without c-ISCS and c-ISCU (Fig. 6D), which is in agreement with the observation from in-gel aconitase assays (Fig 6*B* and 6*C*). Furthermore, reconstitution of aconitase activity increased with time of exposure to reconstituted ISCU (Fig. 6*D*). We then calculated the percentage of converted [4Fe-4S]-IRP1 from apo-IRP1 with increasing concentration of c-ISCU and c-ISCS for reconstitution in order to evaluate the efficiency of [4Fe-4S] cluster transfer. The result showed that IRP1 aconitase activity increased, i.e. more apo-IRP1 was converted into [4Fe-4S]-IRP1, with increasing c-ISCS and c-ISCU concentrations in a 30 min assay (Fig. 6*E*), and approximately 17% of apo-IRP1 could be converted to holoprotein within 30 min. Taken together, reconstitution of IRP1 aconitase activity is a function of the concentration of reconstituted c-ISCU and time of incubation, indicating that both human cytosolic ISCS and ISCU are functional in Fe-S cluster assembly.

DISCUSSION

Iron-sulfur clusters are prosthetic groups that are important in diverse processes, ranging from electron transfer reactions, binding of enzymatic substrates, to regulation of biological activity. In bacteria, important iron-sulfur cluster (ISC) proteins are synthesized within the cytoplasm and iron-sulfur cluster assembly enzymes are mostly encoded by the *isc* operon (1), though in *A. vinelandii*, a separate operon encodes the iron-sulfur cluster assembly enzymes needed for nitrogenase function (21). In eukaryotes, iron-sulfur assembly proteins are found not only in mitochondria, but also in the cytosolic, nuclear and chloroplast compartments (22), and it is important to understand how iron-sulfur proteins acquire their iron-sulfur clusters in physically distinct locations.

In yeast, Lill and colleagues have asserted that all *de novo* assembly of iron-sulfur proteins takes place within the mitochondrial matrix, and that the inner membrane transporter, Atm1, exports iron-sulfur clusters to the cytosol as needed (23). However, in mammals, multiple elaborate post-transcriptional mechanisms involving alternative AUG utilization or alternative splicing have been identified that generate mitochondrial and cytosolic isoforms of proteins involved in Fe-S cluster biogenesis (2) (3) (4), implying that compartmentalization of Fe-S cluster biogenesis in mammalian cells facilitates acquisition of these unstable prosthetic groups at the different subcellular locations where they are needed.

More recently, our hypothesis that iron-sulfur cluster biogenesis occurs in multiple compartments of mammalian cells has been challenged by experiments in which truncated forms of the yeast homologue of ISCS, Nfs1, were used to complement growth defects in *S. cerevisiae*. It was asserted that yeast Nfs1 containing residues 95-497 could restore Fe-S

cluster assembly in yeast, whereas residues 102-497, (which corresponds to the sequence of human c-ISCS) could not. It was hypothesized that a critical tyrosine in the seven residues between residue 95 and residue 102 consisting of the sequence FGTRPIY, was critical for dimerization and function. It was also asserted that tyrosine 101 was present in alignments of all cysteine desulfurases. However, new database searches reveal that proteins assigned as cysteine desulfurases in *Mycobacterium paratuberculosis* (Q9KII6), *Mycoplasma pneumoniae* (P75298) and *M. genitalium* (Q49420) do not contain a tyrosine at a comparable position in the sequence (Fig. 1*A*), suggesting that the tyrosine residue is not strictly conserved as previously suggested (10).

In experiments described in this paper, we demonstrate that human c-ISCS exhibits biochemical and enzymatic properties similar to the prototypical cysteine desulfurases of *A. vinelandii* and *Saccharomyces cerevisiae* Nfs1p, despite its lack of a conserved tyrosine at the N-terminus. Human c-ISCS dimerizes as efficiently as the IscS of *A. vinelandii*, providing evidence that the short sequence motif containing a conserved tyrosine is not critical for dimerization of c-ISCS. Moreover, the cysteine desulfurase specific activity of c-ISCS is close to that of yeast, as demonstrated by measurement of cysteine persulfide formation and enzymatic activity.

In addition, the present study provides *in vitro* evidence for c-ISCU function. Previously, it has been suggested that the cytosolic form of ISCU is non-functional, based on the failure of the human cytosolic ISCU modified by addition of a yeast mitochondrial targeting sequence to rescue growth in yeast (24). It was asserted that the cytosolic isoform of ISCU was the only member of the large Isc/IscU protein family that lacked a highly conserved tyrosine residue at the N-terminus (24). However, we over-expressed and purified cytosolic ISCU, and found that it forms a protein complex with cytosolic ISCS as expected, and that these two proteins together facilitate assembly of the iron-sulfur cluster of IRP1 and yeast mitochondrial aconitase (Fig. 5). Moreover, the cytosolic form of ISCU was as efficient as the mature mitochondrial ISCU in converting apo-IRP1 to a functional aconitase. New database analyses have revealed that numerous members of the ISCU family, including the NifU of *Rhodobacter spheroides* (Q01180), of *Chlamydia pneumoniae* (Q9K1T8), numerous *Mycoplasma* species (Q98R36, Q6KIL2, Q6MTA6, Q8EV25), and *Candidata phytoplasma* asteris (Q6YRB5) lack the tyrosine residue in question (Fig. 1*B*). Finally, RNAi studies have confirmed that human c-ISCU is crucial for Fe-S cluster assembly in IRP1 *in vivo* (13).

In summary, we demonstrate here with multiple assays that the cytosolic forms of ISCS and ISCU in mammalian cells are functional. c-ISCS dimerizes and acquires cysteine persulfide as efficiently as the bacterial prototype protein. Cytosolic forms of ISCS and ISCU form a complex and facilitate *de novo* synthesis of iron-sulfur clusters on both IRP1 and yeast mitochondrial aconitase. Thus, our work here supports the conclusion that cytosolic isoforms of iron-sulfur cluster enzymes are fully functional in mammalian cytosol, and our recent work performing RNAi on mitochondrial vs. cytosolic ISCU isoforms supports that iron-sulfur clusters of cytosolic proteins are synthesized in the cytosolic compartment (13).

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FIGURE LEGENDS

Fig. 1. Alignment of the N-terminus of human c-IscS and c-IscU with IscS/NifS-like (*A*) and IscU/NifU-like (*B*) proteins.

A, The leader sequences of the human, yeast, and mycoplasma pneumoniae IscS are depicted by showing the first three amino acids, followed by the number of unconserved residues not shown, followed by an alignment that shows the initiation codon of the cytosolic form of hIscS with an asterisk. A tyrosine residue (Y) conserved in many species, indicated by a box, is absent in cIscS, as well as in several *mycoplasma* and *mycobacterium* species included in the alignment. *Hs: Homo sapiens, Ec: Escherichia coli, Sc: Saccharomyces cerevisiae, Mpn: Mycobacterium paratuberculosis, Mg: Mycoplasma genitalium, Mpa: Mycoplasma pneumoniae.*

B, The N-termini of multiple IscU sequences, including human c-ISCU are aligned, and a tyrosine residue present in the mitochondrial form, but not in the cytosolic form of ISCU is indicated by a box. Notably, other species, including *Mpu: Mycoplasma pulmonis, Mmo: Mycoplasma mobile, Mpe:Mycoplasma penetrans, Mmy: Mycoplasma mycoides* subsp. mycoides SC*,and Cp:* and *Candidatus Phytoplasma* asteris also lack the tyrosine present in yeast and other bacterial species.

Fig. 2. Overexpression of human cytosolic ISCS (*c-ISCS*), cytosolic ISCU (*c-ISCU*), and human iron regulatory protein 1 (*IRP1*) in *Pichia Pastoris*.
A, Expression of c-ISCS, c-ISCU, or IRP1 was induced in yeast and overexpressed protein was detected in Western blots probed with antibodies against ISCS, ISCU, and IRP1, respectively. *Lane* 1: *Pichia* lysate before induction; lane 2: *Pichia* lysate after induction; lane 3: Human HeLa cell lysate. Mitochondrial (*m-ISCS* and *m-ISCU*) and cytosolic (*c-ISCS* and *c-ISCU*) isoforms of ISCS and ISCU in HeLa cell lysates are indicated. An unprocessed form of m-ISCU previously described (3) is indicated with an asterisk (∗).
B, Over-expressed c-ISCS was purified from *Pichia* lysate using an initial ammonium sulfate ($AMSO_4$) precipitation, followed by collection of DEAE column flowthrough, and subsequent elution from a heparin sepharose column.

Fig. 3. c-ISCS and c-ISCU form a complex upon *in vitro* mixing. Lysates from *Pichia* in which either c-ISCS or c-ISCU was overexpressed were incubated at 4ºC for more than 3 hours, either separately (*top two panels*) or mixed together (*bottom two lanes*) and the individual or mixed lysates were then analyzed in fractions eluted from a size-exclusion FPLC column. Western blots were performed to detect the distribution of c-ISCS and c-ISCU by probing with antibodies to ISCS or ISCU. Purified c-ISCS was also analyzed on the same column. The numbers in the top row show the molecular weight of standard proteins and the numbers in the second row are the FPLC fraction numbers. Note that c-ISCU comigrates with c-ISCS when it can interact with c-ISCS in a large complex of approximately 440kDa (*bottom panel*), but c-ISCU migrates at a very different position, between 25 and 67kD in the gradient when c-ISCS is not added. In contrast,to c-ISCS in *Pichia* lysate, purified c-ISCS migrates around the monomer and dimer size (43-86 kDa).

Fig. 4. c-ISCS is a functional cysteine desulfurase that dimerizes efficiently.
A, Yeast cells that over-express human c-ISCS exhibited increased cysteine desulfurase activity. Cysteine desulfurase activity of cell lysate (80-100 μg total protein) from *Pichia* that over-express IRP1, measured by the sulfide detection assay, was arbitrarily set at 1 to represent the endogenous cysteine desulfurase activity of *Pichia Pastoris*. Lysates from cells that over-expressed c-ISCS alone, or co-over-express c-ISCS and IRP1 are shown in comparison. Lysates from cells that contained the integrated empty plasmid were also measured for comparison. Data represent the average from three independent experiments.
B, Purified c-ISCS forms dimers that are diminished by addition of GSH to samples separated on a non-reducing gel (4-20%) (*lanes 1 and 2*), and cysteine persulfide formation is associated with both monomeric and dimeric forms similar to those formed by other cysteine desulfurases (reviewed in 1), as evidenced by sulfur transfer from ^{35}S-cysteine to c-ISCS (*lane 3*) as described in experimental procedures.
C, Dimerization of c-ISCS and formation of cysteine persulfide are as efficient in c-ISCS as in the standard cysteine desulfurase of *Azotobacter vinelandii* (*Av IscS*). Purified c-ISCU was used as a negative control for specific sulfur transfer. MW markers (SeeBlue® plus 2, invitrogen) (*lane 1*), purified c-ISCU (*lane 2, 5*), c-ISCS (*lane 3, 6*), and *Av* IscS (*lane 4, 7*) were separated on the same gel system as in *B* after incubation with ^{35}S cysteine. The left panel was Coomassie stained, whereas the right panel represents the scan of PhosphoImage exposure, performed with portions of the same samples that are in the left-hand panel, after reaction with ^{35}S cysteine. c-ISCU showed no cysteine persulfide bond formation with ^{35}S in 30 sec. (*lane 5*), whereas c-ISCS (*lane 6*) and *Av IscS* (*lane 7*) showed robust ^{35}S labeling in 30 sec.

Fig. 5. Human c-ISCS and c-ISCU cooperate to facilitate Fe-S cluster assembly in aconitases.
A, Overexpressed IRP1 in *Pichia Pastoris* showed some aconitase activity, likely due to activity of endogenous yeast iron-sulfur assembly enzymes. *P. pastoris* lysates (2 ug protein) containing control empty plasmid (lane 1), over-expressed c-ISCS (*lane 2*), c-ISCU (*lane 3*), or IRP1 (*lane 4*) were analyzed by an aconitase in-gel assay.
B, Endogenous *m*-aconitase of *Pichia* and recombinant IRP1 aconitase activity increased markedly when incubated *in vitro* with both c-ISCS and c-ISCU. Yeast lysate (2 ug protein) containing empty plasmid, overexpressed c-ISCS, and/or c-ISCU were combined with lysates from yeast that overexpressed IRP1 (2 μg protein) and were incubated in 30 μl of 40 mM KCl, 25 mM Tris pH 7.5 for 3 hours in an anaerobic chamber. Mixtures were then assayed by the in-gel aconitase assay. Equal amounts of over-expressed IRP1 in lysate were added to each experimental point, as indicated by the IRP1 Western in the bottom gel panel. The bar-graphs of the lower panel represent quantifications of IRP1 (*IRP1-aco*) or m- aconitase (*m-aco*) activities from the duplicates of the aconitase gel shown above.
C, Comparison of the efficacy of the cytosolic vs. mitochondrial ISCU scaffold isoforms, c-ISCU and m-ISCU, in enhancing transfer of Fe-S clusters to apo-IRP1. *In vitro* reconstitution of the Fe-S clusters of c-ISCU or m-ISCU was performed as outlined in Fig. 6A, with purified reagents, except that apo-IRP1 was added directly (the Mono-Q column was omitted), and the mix was analyzed by the in-gel aconitase assay. The second and third panels show the efficacy of m-ISCU (top panel) and c-ISCU (bottom panel) as a scaffold after reconstitution followed by incubation with apo-IRP1 at three separate time points. The top panel shows that there is negligible reconstitution of IRP1 in the absence of added ISCU. Coomassie staining showed that equal amounts of IRP1 or/and ISCU were added. The graph below the panels represents the relative IRP1 aconitase activity acquired through conversion of apo-IRP1 into [4Fe-4S]-IRP1, enhanced by c-ISCU compared to m-ISCU. The inset shows a Coomassie stained panel of over-expressed c-ISCU and m-ISCU, and reveals that the mature mitochondrial ISCU migrates as a slightly smaller protein, as predicted by the open reading frames of the alternate splice forms, and consistent with the sizes of ISCU isoforms detected in HeLa cells in figure 1.

Fig. 6. Purified human cytosolic forms of ISCS and ISCU reconstitute the Fe-S cluster of apo-IRP1.
A, Schematic outline of experiments performed for figure 6*B-D*. *In vitro* reconstitution of the Fe-S cluster of c-ISCU was performed by incubation of cysteine (2 mM), iron (0.2 mM), and DTT (5 mM) with or without purified c-ISCS and c-ISCU in the anaerobic chamber for 3 hours. Products were fractionated on a Mono-Q column, and eluted with 200 or 400 mM NaCl in 25 mM Tris pH 8.0 buffer. Each fraction was then incubated with equal amounts of apo-IRP1 purified as previously described (11) using an IRE-affinity column to remove any holoprotein present in starting material. Fractions were incubated in an anaerobic chamber, and samples were evaluated by the in-gel aconitase assay (*6B* and *6C*) or evaluated by the $NADP^+$ reduction spectrophotometric assay (*6D* and *6E*).
B, Aconitase activity of IRP1 after the cluster transfer reaction. FT1 and FT2 represent sequential flowthrough fractions from the Mono-Q column. E200-1, E200-2, E400-1, and E400-2 represent eluates from either the first (*E200-1*) or second (*E200-2*) elution with 200 mM NaCl, followed by the first (*E400-1*) or second (*E400-2*) elution with 400 mM NaCl in 25 mM Tris pH 8.0.

C, Western blots were performed to detect the distribution of c-ISCU in relation to IRP1 aconitase activity. Equal addition of apo-IRP1 to each fraction was verified by Western blot. The molar ratio of total ISCU in the E-200-1 lane relative to apo-IRP1 was estimated to be 1.5.
D, Reconstitution of IRP1 aconitase activity in the presence or absence of c-ISCS and c-ISCU was assessed by the $NADP^+$ reduction spectrophotometric assay. Apo-IRP1 was incubated for 30 or 180 min with the E200-1 fraction from the reconstitution reaction containing cysteine, iron and DTT only (- *c-ISCS* - *c-ISCU*) or the E200-1 fraction in which c-ISCS and c-ISCU were present (+ *c-ISCS* + *c-ISCU*). The results represented the average of three independent experiments.
E, Converted [4Fe-4S]-IRP1, assessed by the $NADP^+$ reduction spectrophotometric assay, positively correlates with the concentrations of c-ISCS and c-ISCU. Apo-IRP1 was incubated for 30 min with the mix for the cluster assembly reaction containing cysteine, iron, DTT, and increasing concentrations of c-ISCS and c-ISCU. Mono-Q fractionation was not performed for these experiments.

Figure 1

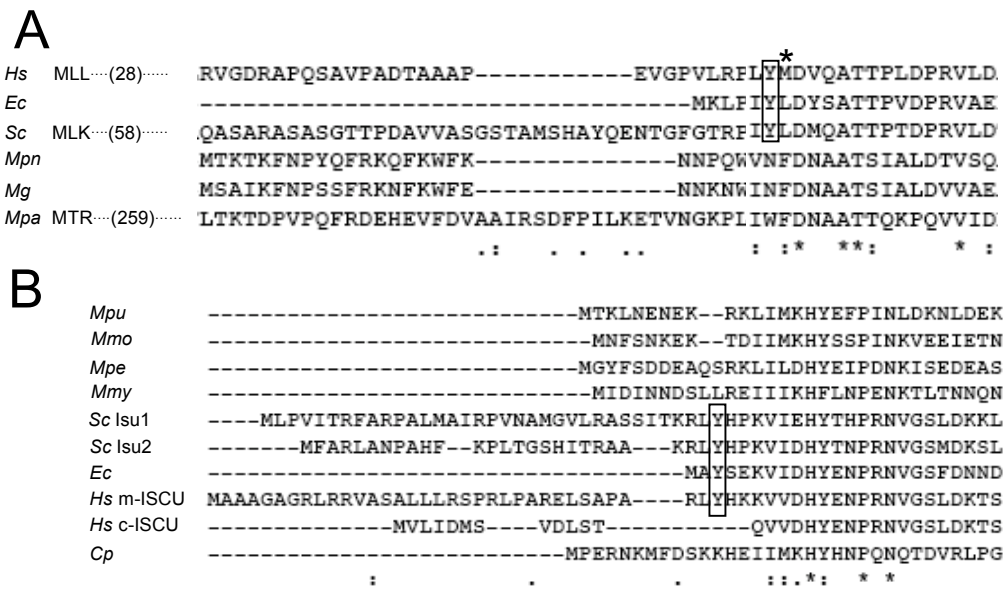
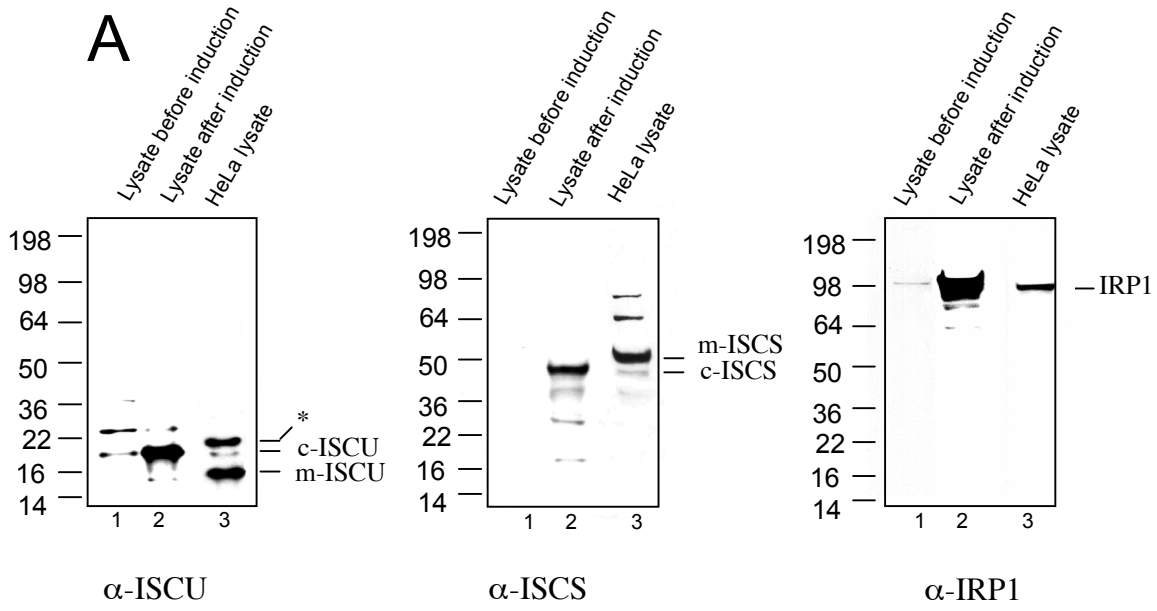

A
Hs MLL····(28)·····
Ec
Sc MLK····(58)·····
Mpn
Mg
Mpa MTR····(259)·····
RVGDRAPQSAVPADTAAAP----------EVGPVLRFLYMDVQATTPLDPRVLD
----------MKLFIYLDYSATTPVDPRVAE
QASARASASGTTPDAVVASGSTAMSHAYQENTGFGTRFIYLDMQATTPTDPRVLD
MTKTKFNPYQFRKQFKWFK----------NNPQWVNFDNAATSIALDTVSQ
MSAIKFNPSSFRKNFKWFE----------NNKNWINFDNAATSIALDVVAE
LTKTDPVPQFRDEHEVFDVAAIRSDFPILKETVNGKPLIWFDNAATTQKPQVVID
B
Mpu
Mmo
Mpe
Mmy
Sc Isu1
Sc Isu2
Ec
Hs m-ISCU
Hs c-ISCU
Cp
----------MTKLNENEK--RKLIMKHYEFPINLDKNLDEK
----------MNFSNKEK--TDIIMKHYSSPINKVEEIETN
----------MGYFSDDEAQSRKLILDHYEIPDNKISEDEAS
----------MIDINNDSLLREIIIKHFLNPENKTLTNNQN
----MLPVITRFARPALMAIRPVNAMGVLRASSITKRLYHPKVIEHYTHPRNVGSLDKKL
-------MFARLANPAHF--KPLTGSHITRAA---KRLYHPKVIDHYTNPRNVGSMDKSL
----------MAYSEKVIDHYENPRNVGSFDNND
MAAAGAGRLRRVASALLLRSPRLPARELSAPA----RLYHKKVVDHYENPRNVGSLDKTS
----------MVLIDMS----VDLST----------QVVDHYENPRNVGSLDKTS
----------MPERNKMFDSKKHEIIMKHYHNPQNQTDVRLPG

Figure 2


A
Lysate before induction
Lysate after induction
HeLa lysate
198
98
64
50
36
22
16
14
1 2 3
*
c-ISCU
m-ISCU
α-ISCU
m-ISCS
c-ISCS
α-ISCS
IRP1
α-IRP1

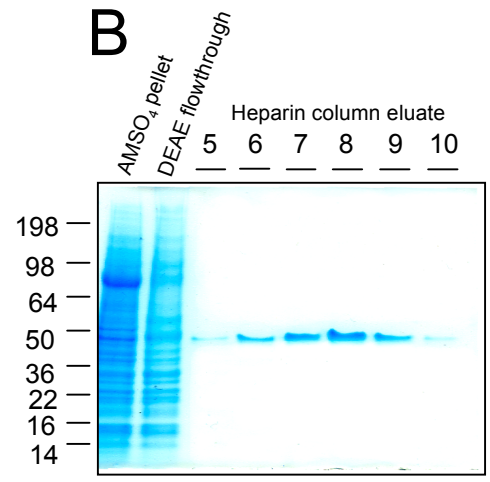

B
AMSO4 pellet
DEAE flowthrough
Heparin column eluate
5 6 7 8 9 10
198
98
64
50
36
22
16
14

Figure 3

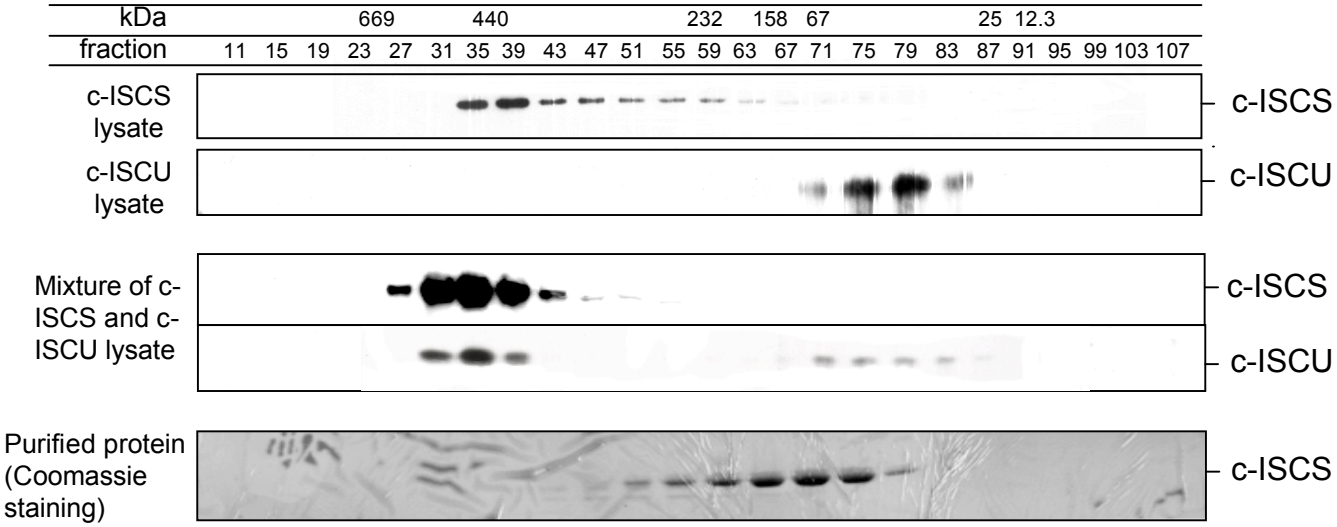

kDa
669
440
232
158
67
25
12.3
fraction
11 15 19 23 27 31 35 39 43 47 51 55 59 63 67 71 75 79 83 87 91 95 99 103 107
c-ISCS lysate
c-ISCS
c-ISCU lysate
c-ISCU
Mixture of c-ISCS and c-ISCU lysate
c-ISCS
c-ISCU
Purified protein (Coomassie staining)
c-ISCS

Figure 4

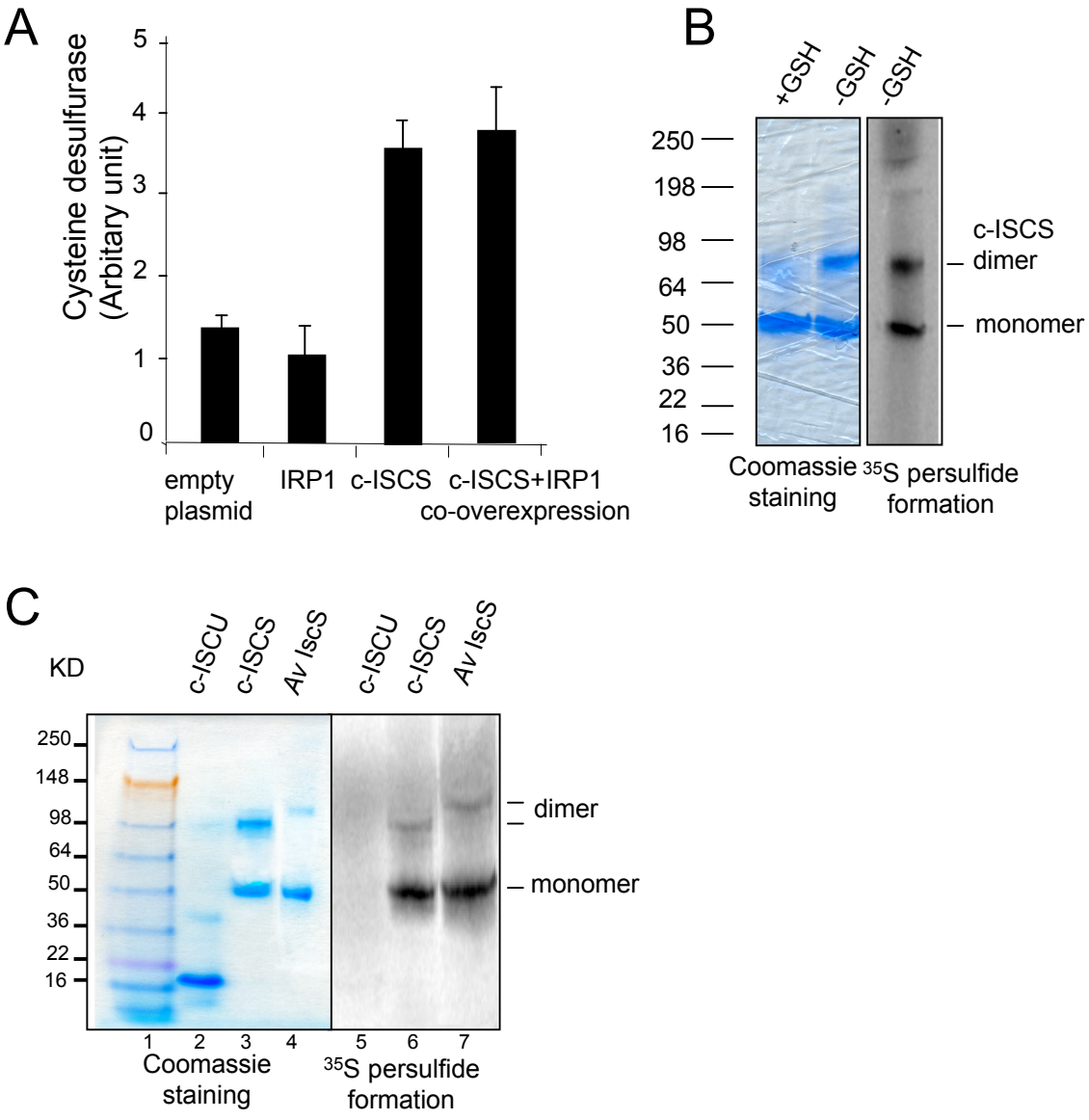

A
Cysteine desulfurase (Arbitary unit)
0
1
2
3
4
5
empty plasmid
IRP1
c-ISCS
c-ISCS+IRP1 co-overexpression
B
+GSH
-GSH
-GSH
250
198
98
64
50
36
22
16
c-ISCS
dimer
monomer
Coomassie staining
35S persulfide formation
C
KD
c-ISCU
c-ISCS
Av IscS
c-ISCU
c-ISCS
Av IscS
250
148
98
64
50
36
22
16
dimer
monomer
1 2 3 4 5 6 7
Coomassie staining
35S persulfide formation

Figure 5

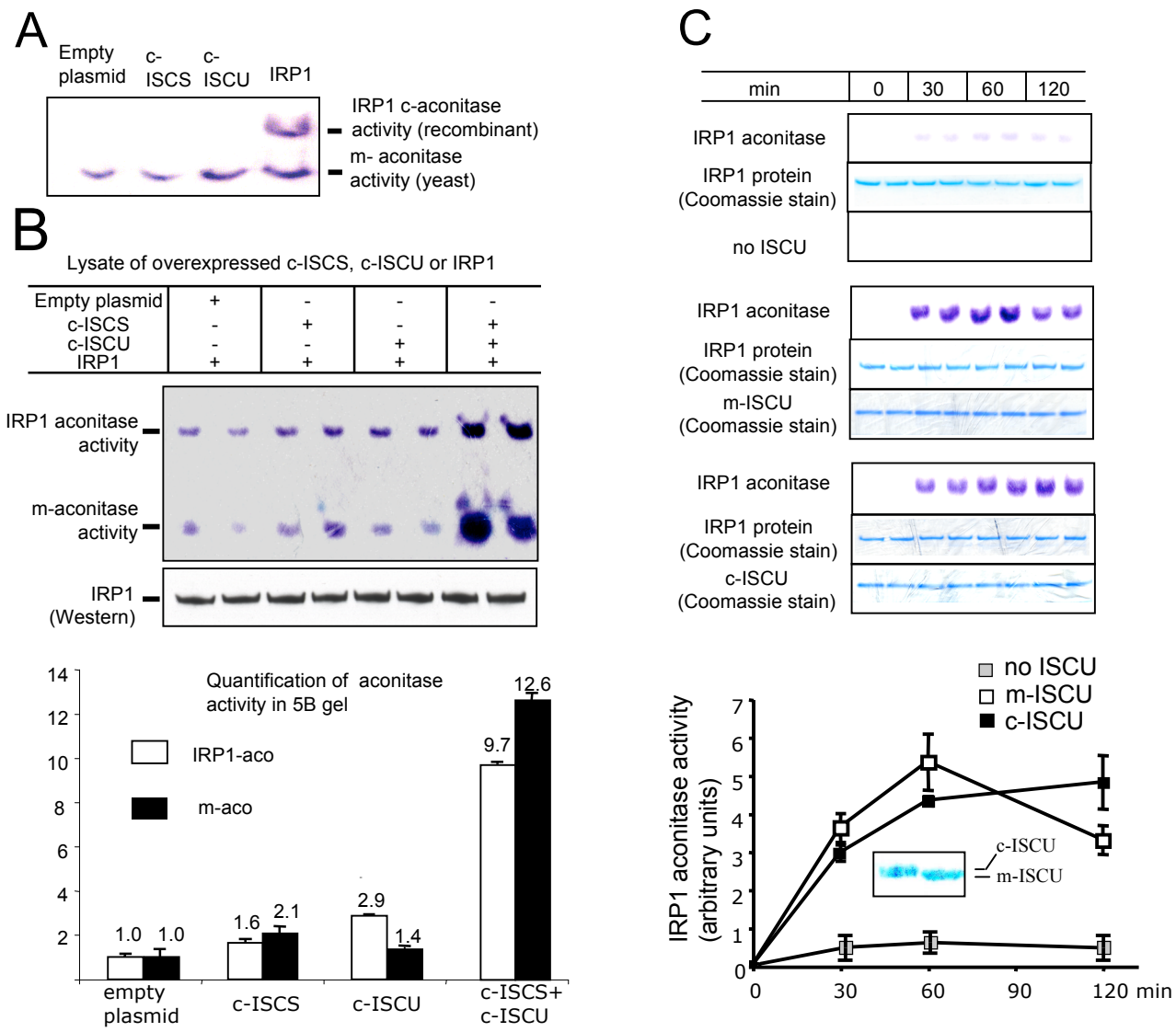
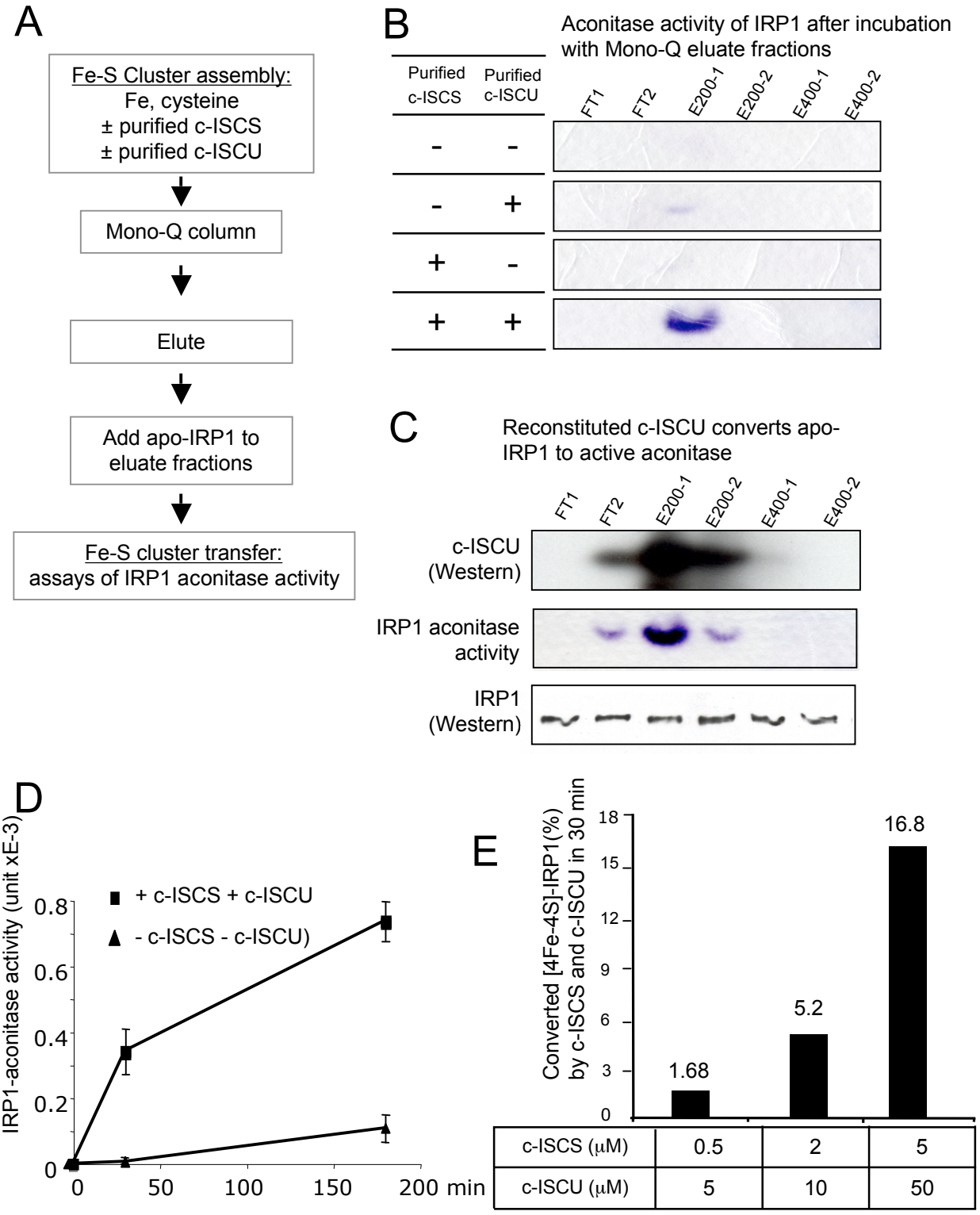

A
Empty plasmid
c-ISCS
c-ISCU
IRP1
IRP1 c-aconitase activity (recombinant)
m- aconitase activity (yeast)
B
Lysate of overexpressed c-ISCS, c-ISCU or IRP1
Empty plasmid + - - -
c-ISCS - + - +
c-ISCU - - + +
IRP1 + + + +
IRP1 aconitase activity
m-aconitase activity
IRP1 (Western)
Quantification of aconitase activity in 5B gel
IRP1-aco
m-aco
1.0 1.0
1.6 2.1
2.9 1.4
9.7 12.6
empty plasmid
c-ISCS
c-ISCU
c-ISCS+ c-ISCU
C
min 0 30 60 120
IRP1 aconitase
IRP1 protein (Coomassie stain)
no ISCU
IRP1 aconitase
IRP1 protein (Coomassie stain)
m-ISCU (Coomassie stain)
IRP1 aconitase
IRP1 protein (Coomassie stain)
c-ISCU (Coomassie stain)
no ISCU
m-ISCU
c-ISCU
IRP1 aconitase activity (arbitrary units)
c-ISCU
m-ISCU
0 30 60 90 120 min

Figure 6


A
Fe-S Cluster assembly:
Fe, cysteine
± purified c-ISCS
± purified c-ISCU
Mono-Q column
Elute
Add apo-IRP1 to eluate fractions
Fe-S cluster transfer:
assays of IRP1 aconitase activity
B
Aconitase activity of IRP1 after incubation with Mono-Q eluate fractions
Purified c-ISCS
Purified c-ISCU
FT1
FT2
E200-1
E200-2
E400-1
E400-2
C
Reconstituted c-ISCU converts apo-IRP1 to active aconitase
c-ISCU (Western)
IRP1 aconitase activity
IRP1 (Western)
D
IRP1-aconitase activity (unit xE-3)
+ c-ISCS + c-ISCU
- c-ISCS - c-ISCU)
0.8
0.6
0.4
0.2
0
0
50
100
150
200 min
E
Converted [4Fe-4S]-IRP1(%) by c-ISCS and c-ISCU in 30 min
18
15
12
9
6
3
0
1.68
5.2
16.8
c-ISCS (μM)
0.5
2
5
c-ISCU (μM)
5
10
50